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STUDY CODE: K06-1120

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FINAL REPORT

CHROMOSOMAL ABERRATION TEST OF

USING CULTURED MAMMALIAN CELLS

February 2006

STATEMENT

I, the undersigned, hereby declare that this report provides a correct English translation of the final report, Study Code K06-1120, issued on February 22, 2006.

March 22, 2006

Date

GLP STATEMENT

Sponsor:

Title: Chromosomal Aberration Test of Cultured Mammalian Cells

Study Code: K06-1120

I, the undersigned, hereby declare that this study was conducted in compliance with "Concerning Standard of the Testing Facilities Conducting the Test Relating to the New Chemical Substances" (Notification No. 1121003 of the Pharmaceutical and Food Safety Bureau, MHLW, No. 3 (November 17, 2003) of the Manufacturing Industries Bureau, METI & No. 031121004 of the Environmental Health Department, MOE (November 21, 2003)) and "OECD Principles of Good Laboratory Practice" (November 26, 1997).

I also confirmed that this report accurately reflected the raw data and the test data were valid.

Study Director: Signed in original February 22, 2006

QUALITY ASSURANCE STATEMENT

Sponsor:

Title: Chromosomal Aberration Test Cultured Mammalian Cells

Study Code: K06-1120

This study was audited and inspected by Quality Assurance Section of The dates audited and/or inspected and the dates reported these results to the study director and the management are as follows.

Items of Inspections/Audits	Dates of Inspections/Audits	Dates of Inspections/Audits Report
Protocol	November 10, 2005	November 11, 2005
Amendment of Protocol	November 10, 2005	November 11, 2005
Preparation of Test Substance (Cell Growth Inhibition Test)	November 10, 2005	November 11, 2005
Treatment of Cells (Cell Growth Inhibition Test)	November 10, 2005	November 11, 2005
Amendment of Protocol (No. 2)	November 15, 2005	November 15, 2005
Amendment of Protocol (No. 3)	November 22, 2005	November 25, 2005
Amendment of Protocol (No. 4)	January 5, 2006	January 6, 2006
Raw Data and Draft Report	January 20, 2006	January 23, 2006
Re-inspection of Raw Data and Draft Report	January 23, 2006	January 24, 2006
Draft Report (Second)	January 27, 2006	January 27, 2006
Re-inspection of Draft Report (Second)	January 30, 2006	January 30, 2006
Final Report	February 22, 2006	February 22, 2006

Therefore, items of inspections below were reported to the study director and the management based on the inspections of institution and another test.

Items of Inspections	Dates of Inspections	Dates of Inspections Report
Preparation and Management of Positive Control Substance	September 5, 2005	January 30, 2006
Collection of Cells and Preparation of Specimens	November 22 and 30, 2005	January 30, 2006
Observation of Specimens	November 22, 2005	January 30, 2006

I, the undersigned, hereby declare that this report provides an accurate description of the methods and procedures used in this study and that the reported results accurately reflect the raw data obtained.

Section Chief, Quality Assurance: Signed in original February 22, 2006

Study Code: K06-1120
Test Substance Code: HR6304
Sponsor Code: A-0080

TITLE

Chromosomal Aberration Test of Cultured Mammalian Cells

SPONSOR

TESTING FACILITY

PURPOSE OF STUDY

The ability of the test substance to induce chromosomal aberrations was examined by using Chinese hamster lung fibroblasts (CHL/IU cells).

TESTING METHOD

This study was conducted in accordance with "III Mutagenicity Test: Chromosomal Aberration Test Using Cultured Mammalian Cells" prescribed in "Concerning Testing Methods Relating to the New Chemical Substances" (Notification No. 1121002 of the Pharmaceutical and Food Safety Bureau, MHLW, No. 2 (November 13, 2003) of the Manufacturing Industries Bureau, METI & No. 031121002 of the Environmental Health Department, MOE (November 21, 2003)) and with "OECD Guidelines for Testing of Chemicals, 473, *In Vitro* Mammalian Chromosome Aberration Test" (July 21, 1997).

GLP COMPLIANCE

This study was conducted in compliance with "Concerning Standard of the Testing Facilities Conducting the Test Relating to the New Chemical Substances" (Notification No. 1121003 of the Pharmaceutical and Food Safety Bureau, MHLW, No. 3 (November 17, 2003) of the Manufacturing Industries Bureau, METI & No. 031121004 of the Environmental Health Department, MOE (November 21, 2003)) and "OECD Principles of Good Laboratory Practice" (November 26, 1997).

PERIOD OF STUDY

Commencement of Study:	November 4, 2005
Initiation of Experiment (Initiation of Treatment of Cell Growth Inhibition Test):	November 10, 2005
Completion of Experiment (Completion of Observation of Specimens):	December 5, 2005
Preparation of Draft Report:	January 25, 2006
Completion of Study:	February 22, 2006

STORAGE AND RETENTION PERIOD OF DATA

The raw data, protocol and amendment, study contract documents, test substance information, final report, other record documents and specimens will be stored in the archive of this testing facility of our organization for a period of 10 years from the date of receipt of the notification that they are applicable to Article 4, Paragraphs 1 or 2, Article 4-2, Paragraphs 2, 3 or 8, Article 5-4, Paragraph 2, Article 24, Paragraph 2 or Article 25-3, Paragraph 2 of the Japanese Chemical Substances Control Law No. 117 (1973). The sponsor will inform this testing facility of our organization of the date of receipt of the notification. After termination of the retention period, any measures taken will be done so with the approval of the sponsor. Any measures that are liable to deteriorate markedly will be retained only for as long as the quality of the preparation permits evaluation with the sponsor's consent.

RETENTION OF ORIGINAL DOCUMENTS

An original protocol, original protocol amendments and an original final report will be retained at the testing facility. The copies of these originals that the study director was proper to be accurate copy were send to the sponsor.

STUDY DIRECTOR AND PERSONS CONCERNED WITH THE STUDY

Study Director:

Persons Concerned with The Study and Their Operation:

(Preparation of test substance formulation, cell treatment and microscopic observation of specimens)

(Microscopic observation of specimens)

(Microscopic observation of specimens)

APPROVAL BY AUTHOR

Study Director: Signed in original February 22, 2006

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SUMMARY

The ability induce chromosomal aberrations was investigated by using Chinese hamster lung fibroblasts (CHL/IU cells).

Based on the results of the cell growth inhibition test, the doses in the chromosomal aberration test were set at 723, 868, 1040, 1250, 1500 and 1800 μg/mL in short-term treatment without S9 mix and at 603, 723, 868, 1040, 1250, 1500 and 1800 μg/mL in short-term treatment with S9 mix.

In observation of specimens in chromosomal aberration test, the highest dose for observation was selected at the minimum dose that the cell growth rate showed below 50%, and the total 3 doses were selected for the observation doses of the test substance. Accordingly, the doses for observation were selected at 1250, 1500 and 1800 μg/mL for short-term treatment without S9 mix and at 868, 1040 and 1250 μg/mL in short-term treatment with S9 mix. In the observation, the frequencies of cells with structural aberrations and of numerical aberration cells were scored.

As a result of observation of specimens, the maximum frequencies of cells with structural aberration was over 10% in the short-term treatments without and with S9 mix, and the frequencies were recognized as a dose-related increase. Therefore, structural aberration was judged to be positive. The frequencies of numerical aberration cells showed below 5% at all doses of the test substance in the short-term treatments without and with S9 mix, therefore, numerical aberration were judged to be negative.

On the other hand, the frequencies of cells with structural aberrations and of numerical aberration cells in the negative control treated for distilled water showed below 5%, and the frequencies of cells with structural aberrations in the positive controls treated with mitomycin C or cyclophosphamide, showed above 20%, indicating the proper performance of the present study.

It is concluded not induce numerical aberration but it induces structural aberration under the present test conditions.

MATERIALS AND METHODS

1. TEST SUBSTANCE AND POSITIVE CONTROL SUBSTANCES

1.1 Test Substance (Information Provided by the Sponsor)

1) Name

Other Name:

CAS No.: —

2) Lot No.

RS4-56

3) Supplier

4) Rational formula

5) Purity

99.5 w/w%

6) Names and concentrations of impurities

Water: 0.5 w/w%

7) Physicochemical properties

Appearance at ordinary temperature: white solid

Molecular weight:

Stability: stable at room temperature

stable in water, DMSO and acetone

Melting point:

Boiling point: —

Vapor pressure: —

Partition coefficient (1- octanol/water): —

Hydrolyzability: —

Solubility: soluble in oil and water

Degree of solubility

Water: ≧363 mg/mL (measured at the testing facility)

DMSO: —

Acetone: —

Others: —

8) Storage conditions

Stored at room temperature (cabinet No. 1 in the test substance storage room, the tolerance temperature: 10-30°C).

9) Precautions

Gloves, a mask, a head cap and a lab coat were worn.

1.2 Positive Control Substances

1) Mitomycin C (MMC, 2 mg/vial)

Manufacturer:	Kyowa Hakko Kogyo Co., Ltd.
Lot No.:	440ADE
Appearance:	royal purple powder
Content:	101%
Grade:	for injection

2) Cyclophosphamide monohydrate (CPA)

Manufacturer:	Wako Pure Chemicals Industries, Ltd.
Lot No.:	PKQ7031
Appearance:	white crystals or crystalline powder
Content:	99.0%
Grade:	for biochemistry

3) Storage conditions

MMC was stored at room temperature (cabinet No. 2 in the test substance storage room, the tolerance temperature: 10-30°C) and CPA in a cold dark place (refrigerator No.13 in the test substance storage room, the tolerance temperature: 1-10°C).

4) Precautions

Gloves, a mask, a head cap and a lab coat were worn.

2. CELLS

2.1 Cell Line and Reason for Selection

Chinese hamster lung fibroblasts (CHL/IU cells) were supplied by Health Science Research Resources Bank, Japan Health Sciences Foundation on April 17, 2002. The modal number of chromosomes was 25 per cell. The doubling time was about 15 hours. It was confirmed in the testing facility that the cells were mycoplasma free and the frequency of the spontaneous chromosomal aberration was below 5%.

CHL/IU cells have been recommended the availability on *in vitro* chromosome aberration test in "Concerning Testing Methods Relating to the New Chemical Substances" and "OECD Guidelines for Testing of Chemicals, 473, *In Vitro* Mammalian Chromosome Aberration Test".

2.2 Storage

Cells were suspended in medium [Eagle's minimum essential medium (Nissui Pharmaceutical Co., Ltd.) and 10 vol% heat-inactivated newborn calf serum (NBCS, Sanko Junyaku Co., Ltd.)] including 10 vol% DMSO and were frozen in liquid nitrogen.

2.3 Culture Condition

Cells were cultured in a CO_2 incubator (MCO-345, SANYO Electric Co., Ltd.) which was set at 37°C and 5% CO_2 under humid condition.

2.4 Subculture

Cells were subcultured in 90-mm diameter Petri dishes (Nunc A/S) twice a week. Passage number of cells was at 21 for the cell growth inhibition test and 4 for the chromosomal aberration test after receipt.

3. MEDIUM AND S9 MIX

3.1 Medium

L-glutamine (final concentration: 0.292 g/L) and sodium hydrogen carbonate (final concentration: approximately 1.85 g/L) were added to Eagle's minimum essential medium (Lot No. 535508, Nissui Pharmaceutical Co., Ltd.). This medium was then supplemented with 10 vol% heat-inactivated NBCS (Lot No. 27020859, Sanko Junyaku Co., Ltd.).

3.2 S9 Mix

1) Rat liver S9

S9 (Lot No. 05080505, manufactured on August 5, 2005, protein content: 22.1 mg/mL, Oriental Yeast Co., Ltd.) which was prepared from livers of 7-week-old male SD rat (body weight of rats: 212.4±9.5 g) administered a combination of phenobarbital and 5,6-benzoflavone was used. S9 was frozen and preserved in an ultra-deep freezer (MDF-U481ATR, SANYO Electric Co., Ltd, the tolerance temperature: below -80°C) until use. S9 was used within six months after the day of manufacturing.

2) Composition of S9 mix

One milliliter of S9 mix consisted of 0.3 mL S9, 5 μmol $MgCl_2$, 33 μmol KCl, 5 μmol G-6-P, 4 μmol NADP and 4 μmol HEPES (pH 7.2) and S9 mix was prepared just prior to use and was stored in ice until use.

4. CELL PRE-CULTURE

A 60-mm diameter plastic dish (Asahi Techno Glass Corporation) was used for cell culture. Five milliliters of a cell suspension of 5×10^3 cells/mL seeded into a dish in

the cell growth inhibition test and the chromosomal aberration test was cultured continuously for 3 days.

5. PREPARATION OF TEST SUBSTANCE FORMULATION AND POSITIVE CONTROL SUBSTANCE SOLUTIONS

5.1 Preparation of Test Substance Formulation

1) Solvent

Distilled water (for injection, Lot No. K3G76, Otsuka Pharmaceutical Factory Inc.)

2) Reason for selection of solvent

The test substance was soluble at 363 mg/mL in distilled water. The results showed that the test substance formulation at 363 mg/mL in distilled water was not indicated any change in color nor exothermic at room temperature within 2 hours after preparation. Therefore, distilled water was selected in a solvent.

3) Preparation method

After the test substance was weighed, it was dissolved using a mixer and the test substance formulation was prepared with distilled water. The test substance formulation was diluted with distilled water and the test substance formulations of 100 times concentration of the test substance in the medium were prepared. It was prepared without correcting purity of the test substance because the purity of the test substance was above 95%.

4) Preparation time

The test substance formulations were prepared immediately before use, stored at room temperature and used within 0.5 hour after preparation.

5.2 Preparation of Positive Control Substance Solutions

1) Preparation method and storage

MMC and CPA were dissolved in distilled water at 0.01 mg/mL and 1 mg/mL, respectively. The positive control substance solutions were frozen in an ultra-deep freezer (MDF-U481ATR, SANYO Electric Co., Ltd, the tolerance temperature: below -80°C).

2) Preparation time and expiry in use

The positive control substance solutions were thawed at the time of use and used within 1 hour. The stock solutions were used within 6 months after preparation.

6. TEST PROCEDURE

6.1 Cell Growth Inhibition Test

1) Procedure

For the short-term treatment without S9 mix, the medium was removed from a pre-culture, and the cells were treated for 6 hours in well mixed medium containing 30 μL of the test substance formulation or the solvent and 3 mL of the fresh treatment medium. For the short-term treatment with S9 mix, the medium was removed from a pre-culture, and the cells were treated for 6 hours in well mixed treatment medium consisting of 0.5 mL of S9 mix and 30 μL of the test substance formulation or the solvent and 2.5 mL of the fresh treatment medium. After treatment, the medium was removed, and the cells were rinsed 3 times with 2 mL of Dulbecco's physiological phosphate buffered solution without Ca^{2+} and Mg^{2+}. Cells were then cultured for another 18 hours in 5 mL of fresh medium.

For the continuous treatment, the medium was removed from a pre-culture, and the cells were treated for 24 hours with well mixed medium containing 50 μL of the test substance formulation or the solvent and 5 mL of the fresh treatment medium.

In the short-term and the continuous treatment methods, 50 μL of a 10 μg/mL demecolcine solution was added to each dish 2 hours before the end of the culture.

At the start and the end of the treatment, and at the end of the culture, precipitation of the test substance, the color change of the medium and the corrosion of the dish were observed with the unaided eyes.

At the end of the culture, a cell suspension was prepared to collect from each dish by a treatment with 0.25 w/v% trypsin of 2 mL. After 200 μL of the cell suspension was diluted with 10 mL of Cell Pack (Sysmex Corporation), the number of the cells was measured by using a Microcell counter (CDA-500, Sysmex Corporation), and the cell growth rate and the 50% cell growth inhibition concentration (IC_{50}) were calculated. The IC_{50} was obtained from a linear line drawn between 2 plots; the one being greater and the other lower than, and both closest to 50% of the cell growth rate.

Cells were then collected by a centrifugation at 1000 rpm (185×g) for 5 minutes and were treated hypotonically with 3 mL of 0.075 mol/L KCl at 37°C for 15 minutes. Following the hypotonic treatment, the cells were pre-fixed once with approximately 0.3 mL of a fixative solution (methanol : acetic acid = 3 : 1), and were completely fixed over two times with 3 mL of fixative solution. Then, the

cell suspension was prepared with a fixative solution, two drops of the suspension were placed on a slide glass, and stained about 15 minutes with 2 vol% Giemsa solution in 1/15 mol/L phosphate buffer solution (pH6.8). One specimen was prepared per dose.

2) Dose levels

In each treatment method, the highest dose was set at 3630 μg/mL equivalent to 10 mmol/L in accordance with the guidelines, and 8 lower doses, 14.2, 28.4, 56.7, 113, 227, 454, 908 and 1820 μg/mL were set based on a geometric progression of 2. In each treatment method, duplicate dishes were used for each dose.

3) Observation and scoring

Specimens were observed to check the presence or absence of mitotic metaphases, and the frequency of the cells with chromosomal aberration was calculated by observating on 50 metaphases per dose at which the dose setting of chromosomal aberration test was considered to be referred.

(1) Structural aberration

The number of metaphases with structural aberrations excluding gaps was recorded. Gaps were defined as an achromatic region smaller than the width of one chromatid.

(2) Numerical aberration

The number of polyploid showing triploid or more was scored.

6.2 Chromosomal Aberration Test

1) Procedure

Chromosomal aberration test was carried out using the same procedure as that of the cell growth inhibition test, with the following positive controls. Four specimens per dose were prepared. Because a positive result was predicted for the cell growth inhibition test in short-term treatments, the chromosomal aberration test in the short-term treatments was only conducted and the chromosomal aberration test in the 24 hours continuous treatment was not conducted.

Treatment method		Substance	Dose
Short-term treatment	Without S9 mix	MMC	0.1 μg/mL
	With S9 mix	CPA	6 μg/mL

In the short-term treatment without S9 mix, 30 μL of a 0.01 mg/mL MMC solution was added to each dish. In the short-term treatment with S9 mix, 18 μL of a 1 mg/mL CPA solution was added to each dish.

2) Setting doses of test substance

From the results of the cell growth inhibition test in the short-term treatments without and with S9 mix, a cytotoxicity of the test substance that the cell growth rate showed below 50% compared with the negative controls was obtained. IC_{50} was calculated with 1400 μg/mL in short-term treatment without S9 mix and 1100 μg/mL in short-term treatment with S9 mix. Therefore, the highest dose in the chromosomal aberration test was selected at 1800 μg/mL, and 6 or 7 doses were set based on a geometric progression of 1.2 for each treatment method as follows. Duplicate dishes were tested for each dose.

Treatment method		Setting dose
Short-term treatment	Without S9 mix	723, 868, 1040, 1250, 1500 and 1800 μg/mL
	With S9 mix	603, 723, 868, 1040, 1250, 1500 and 1800 μg/mL

3) Observation

(1) Dose for observation

All specimens of the negative and the positive controls set as the control groups were observed.

In the short-term treatment without and with S9 mix, a cytotoxicity of the test substance that the cell growth rate showed below 50% compared with the negative controls was obtained and the chromosome observation was enable at the minimum dose that the cell growth rate showed below 50%. Therefore, the highest dose for observation was selected at the minimum dose that the cell growth rate showed below 50%, and the total 3 doses were selected for the observation doses of the test substance. Accordingly, the highest dose was selected at 1800 μg/mL in the short-term treatment without S9 mix and at 1250 μg/mL in the short-term treatment with S9 mix, and 3 doses were set as follows.

Treatment method		Dose for observation
Short-term treatment	Without S9 mix	1250, 1500 and 1800 μg/mL
	With S9 mix	868, 1040 and 1250 μg/mL

All specimens observed were randomly coded and observed in a blinded manner.

(2) Structural aberration

Two hundred metaphase cells per dose (50 cells per specimen) containing 25±2 chromosomes were scored using a microscope. The frequency of cells with structural aberrations and the number of their composition in each aberration were recorded. Gaps were defined as an achromatic region smaller than the width of one chromatid and were recorded separately from the structural aberrations.

(3) Numerical Aberration

The numbers of polyploid (cell with 38 or more chromosomes) cells among 200 metaphase cells per dose (50 cells per specimen) observed were recorded.

6.3 Confirmation Test

In the short-term treatments, the frequencies of cells with structural aberration in the test substance treatment groups showed 10% or more indicating a dose-dependence, which meant a positive result, therefore, a confirmation test was not conducted.

7. JUDGEMENT CRITERIA OF RESULTS

The findings were judged to be positive when the frequencies of cells with structural aberrations showed 10% or more with a dose-dependence or the frequencies of cells with structural aberrations showed 5% or more both in the chromosomal aberration test and the confirmation test. The other cases were judged to be negative. No statistical analyses were used. The frequencies of numerical aberration cells were judged according to the same criteria as that of the structural aberration. D_{20} value indicating a concentration which will induce chromosomal aberration of 20% was calculated.

8. VALIDITY OF TEST

This study was regarded as valid as follows: 1) the frequencies of cells with chromosomal aberrations did not fluctuate markedly between two culture dishes, 2) the frequencies of cells with aberrations in the negative control showed below 5%, and 3) the frequencies of cells with structural aberrations excluding gaps in the positive controls showed 20% or more.

FACTORS AFFECTED RELIABILITY OF TEST

There were no other factors which might affect the reliability of the test.

TEST RESULTS

1. CELL GROWTH INHIBITION TEST (Table 1 and Fig. 1)

The IC_{50} was calculated with 1400 μg/mL in the short-term treatment without S9 mix and 1100 μg/mL in the short-term treatment with S9 mix and 430 μg/mL in the 24 hours continuous treatment.

In each treatment method, precipitation of the test substance was not observed at all doses for each treatment method. Color change of the medium and the corrosion of

the culture dish were not observed at all doses in the short-term treatments without and with S9 mix, but color of medium was slightly turned to red at 227 μg/mL or more in 24 hours continuous treatment.

In the short-term treatments without and with S9 mix, the frequencies of numerical aberration cells were below 5% at all observation doses but the maximum frequencies of cells with structural aberrations showed 18% and 22%, and the increase of structural aberration was shown.

2. CHROMOSOMAL ABERRATION TEST

2.1 Short-term Treatment (Tables 2, 3 and Figs. 2, 3)

1) Without S9 mix

(1) Cell growth rate and IC_{50}

The cell growth rates at 723, 868, 1040, 1250, 1500 and 1800 μg/mL of the test substance were 88.1, 83.2, 84.3, 71.7, 59.6 and 35.1%, respectively. The IC_{50} was calculated with 1600 μg/mL.

(2) Precipitation of the test substance, color of medium and corrosion of culture dish

Precipitation of the test substance, color change of the medium and the corrosion of the culture dish were not observed at the start and the end of the treatment and at the end of the culture.

(3) Frequency of cells with structural aberrations

The frequencies were 0.5% in the negative control and 69.0% in the positive control with MMC. The frequencies of cells with structural aberrations at 1250, 1500 and 1800 μg/mL were 3.0, 8.5 and 15.0%, respectively, which was 10% or more at maximum and the frequencies were recognized as a dose-related increase, therefore, the results were judged to be positive.

(4) Frequency of numerical aberration cells

The frequencies of numerical aberration cells were below 5.0% at all doses including the negative and the positive controls, respectively, therefore, the results were judged to be negative.

(5) D_{20} value

D_{20} value for structural aberration was calculated with 2.2 mg/mL.

2) With S9 mix

(1) Cell growth rate and IC_{50}

The cell growth rates at 603, 723, 868, 1040, 1250, 1500 and 1800 μg/mL of the test substance were 82.8, 79.7, 73.6, 60.9, 47.6, 34.5 and 12.2%, respectively. The IC_{50} was calculated with 1200 μg/mL.

(2) Precipitation of the test substance, color of medium and corrosion of culture dish

Precipitation of the test substance, color change of the medium and the corrosion of the culture dish were not observed at the start and the end of the treatment and at the end of the culture.

(3) Frequency of cells with structural aberrations

The frequencies were 3.0% in the negative control and 57.5% in the positive control with CPA. The frequencies of cells with structural aberrations at 868, 1040 and 1250 μg/mL were 1.5, 8.0 and 17.5%, respectively, which was 10% or more at maximum, and the frequencies were recognized as a dose-related increase, therefore, the results were judged to be positive.

(4) Frequency of numerical aberration cells

The frequencies of numerical aberration cells were below 5.0% at all doses including the negative and the positive controls, respectively, therefore, the results were judged to be negative.

(5) D_{20} value

D_{20} value for structural aberration was calculated with 1.5 mg/mL.

3. TYPICAL PHOTOS OF CHROMOSOMAL ABERRATIONS

Normal cell and cell with structural aberrations were shown in photos 1 and 2, respectively.

4. RESULTS OF OBSERVATION OF CHROMOSOMES IN ACCORDANCE WITH "CONCERNING TESTING METHODS RELATING TO THE NEW CHEMICAL SUBSTANCES" AND BACKGROUND DATA (APPENDICES 1 AND 2)

The results of observation of chromosomes in accordance with "Concerning Testing Methods Relating to the New Chemical Substances" and background data of the negative and the positive controls in the testing facility were attached to appendix 1 and appendix 2, respectively.

DISCUSSION AND CONCLUSION

In each treatment method, the frequencies of cells with chromosomal aberrations did not fluctuate markedly between two culture dishes, and the frequencies of cells with aberrations were below 5% in the negative controls, and the frequencies of cells with structural aberrations excluding gaps were over 20% in the positive controls, indicating that the present study was appropriately performed.

As a result of chromosomal aberration test for the short-term treatments without and with S9 mix, the maximum frequency of cells with structural aberrations was over 10%, and the frequencies were recognized as a dose-related increase, therefore, structural aberration was judged to be positive. On the other hand, the frequencies of cells of numerical aberration cells were below 5% at all observation doses of the test substance. Consequently, numerical aberration was judged to be negative.

Based on the above results, it was considered : induce numerical aberrations but induced structural aberration under the present test conditions.

REFERENCES

1. Toshio Sofuni (ed.) (1999) Data book of chromosomal aberration test *in vitro*. Revised edition, 1998 (in Japanese). Life-science Information Center.
2. Japan Environmental Mutagenicity Society/Mammalian Mutagenicity Study Group (ed.) (1988) Atlas of chromosomal aberration induced with chemical substance (in Japanese). Asakura Publishing Co., Tokyo.

Table 1 Results of the cell growth inhibition test

Substance	Dose (μg/mL)	Treatment-recovery time (hour)	S9 mix	Cell growth rate (%)	Precipitation of test substance in medium [a]			Frequency of cells with aberrations (%)[b]	
					Treatment start	Treatment end	Culture end	Structural aberration	Numerical aberration
Distilled water	0	6-18	-	100	-	-	-	0	0
	14.2	6-18	-	107.9	-	-	-	n.o.	n.o.
	28.4	6-18	-	93.5	-	-	-	n.o.	n.o.
	56.7	6-18	-	101.6	-	-	-	n.o.	n.o.
	113	6-18	-	90.1	-	-	-	n.o.	n.o.
	227	6-18	-	91.6	-	-	-	0	0
	454	6-18	-	76.6	-	-	-	0	0
	908	6-18	-	76.7	-	-	-	2	0
	1820	6-18	-	28.6	-	-	-	18	0
	3630	6-18	-	0.6	-	-	-	no meta	
				IC_{50} 1400 μg/mL					
Distilled water	0	6-18	+	100	-	-	-	0	0
	14.2	6-18	+	94.8	-	-	-	n.o.	n.o.
	28.4	6-18	+	80.9	-	-	-	n.o.	n.o.
	56.7	6-18	+	81.6	-	-	-	n.o.	n.o.
	113	6-18	+	76.3	-	-	-	n.o.	n.o.
	227	6-18	+	71.2	-	-	-	0	0
	454	6-18	+	59.8	-	-	-	0	0
	908	6-18	+	61.0	-	-	-	6	0
	1820	6-18	+	14.6	-	-	-	22	2
	3630	6-18	+	2.1	-	-	-	no meta	
				IC_{50} 1100 μg/mL					
Distilled water	0	24-0	-	100	-	-		n.o.	n.o.
	14.2	24-0	-	108.7	-	-		n.o.	n.o.
	28.4	24-0	-	106.7	-	-		n.o.	n.o.
	56.7	24-0	-	99.0	-	-		n.o.	n.o.
	113	24-0	-	83.6	-	-		n.o.	n.o.
	227	24-0	-	63.1	-	-[c]		n.o.	n.o.
	454	24-0	-	48.4	-	-[c]		n.o.	n.o.
	908	24-0	-	50.1	-	-[c]		n.o.	n.o.
	1820	24-0	-	2.1	-	-[c]		no meta	
	3630	24-0	-	3.2	-	-[c]		no meta	
				IC_{50} 430 μg/mL					

n.o.: not observed, no meta: metaphases were not observed.

a) Precipitation of the test substance: -, absence; +, presence

b) The frequency of cells with chromosomal aberrations was calculated by observating 50 metaphases per dose.

c) Color of medium was slightly turned to red.

The dose was set at 3630 μg/mL as a 10 mmol/L of the test substance, which was the maximum dose in the guidelines, the dose levels based on a geometric progression of 2 were selected.

Table 2 Results of chromosomal aberration test

Substance	Dose (μg/mL)	Treatment-recovery time (hour)	S9 mix	Cell growth rate (%)	Precipitation of test substance in medium [a]			Frequency of cells with aberrations (%)[b]	
					Treatment start	Treatment end	Culture end	Structural aberration	Numerical aberration
Distilled water	0	6-18	-	100	-	-	-	0.5	0.0
	723	6-18	-	88.1	-	-	-	n.o.	n.o.
	868	6-18	-	83.2	-	-	-	n.o.	n.o.
	1040	6-18	-	84.3	-	-	-	n.o.	n.o.
	1250	6-18	-	71.7	-	-	-	3.0	1.0
	1500	6-18	-	59.6	-	-	-	8.5	0.5
	1800	6-18	-	35.1	-	-	-	15.0	0.5
MMC	0.1	6-18	-	ND	n.o.	n.o.	n.o.	69.0	0.0
			IC_{50}	1600 μg/mL			D_{20}	2.2 mg/mL	-
Distilled water	0	6-18	+	100	-	-	-	3.0	1.5
	603	6-18	+	82.8	-	-	-	n.o.	n.o.
	723	6-18	+	79.7	-	-	-	n.o.	n.o.
	868	6-18	+	73.6	-	-	-	1.5	0.0
	1040	6-18	+	60.9	-	-	-	8.0	0.0
	1250	6-18	+	47.6	-	-	-	17.5	0.0
	1500	6-18	+	34.5	-	-	-	n.o.	n.o.
	1800	6-18	+	12.2	-	-	-	n.o.	n.o.
CPA	6	6-18	+	ND	n.o.	n.o.	n.o.	57.5	0.0
			IC_{50}	1200 μg/mL			D_{20}	1.5 mg/mL	-

MMC: mitomycin C, CPA: cyclophosphamide monohydrate

ND: not detected

n.o.: not observed

a) Precipitation of the test substance: -, absence; +, presence

b) The frequency of cells with chromosomal aberrations was calculated by observating 200 metaphases per dose.

Table 3 Results of chromosomal aberration test (short-term treatment)

Name of test substance : .

K06-1120

Treatment time (h)	S9 mix	Dose (μg/mL)	Number of structural chromosomal aberrations (Average)						Total number of cells with structural aberrations (frequency%)	Number of gaps (frequency%)	Cell growth rate (%)	Number of cells with numerical chromosomal aberrations (frequency%)			
			Number of cells observed	Chromatid break	Chromatid exchange	Chromosome break	Chromosome exchange	Others				Number of cells observed	Polyploids	Others	Total number of cells with aberrations
6 - 18	−	Negative control (D.W.) 0	100	0	0	0	0	0	0	3	100	100	0	0	0
			100	2	0	0	0	0	1	0		100	0	0	0
			200	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	3 (1.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	−	1250	100	0	1	0	0	0	1	0	72.1	100	1	0	1
			100	4	0	0	1	0	5	0	71.2	100	1	0	1
			200	4 (2.0)	1 (0.5)	0 (0.0)	1 (0.5)	0 (0.0)	6 (3.0)	0 (0.0)	(71.7)	200	2 (1.0)	0 (0.0)	2 (1.0)
6 - 18	−	1500	100	7	8	0	0	0	12	0	55.3	100	0	0	0
			100	7	9	0	0	0	5	0	63.9	100	1	0	1
			200	14 (7.0)	17 (8.5)	0 (0.0)	0 (0.0)	0 (0.0)	17 (8.5)	0 (0.0)	(59.6)	200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	−	1800	100	5	15	0	0	0	11	0	31.2	100	0	0	0
			100	11	15	1	0	0	19	0	39.0	100	1	0	1
			200	16 (8.0)	30 (15.0)	1 (0.5)	0 (0.0)	0 (0.0)	30 (15.0)	0 (0.0)	(35.1)	200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	−	Positive control (MMC) 0.1	100	49	81	0	0	1	71	1		100	0	0	0
			100	56	74	0	0	0	67	2		100	0	0	0
			200	105 (52.5)	155 (77.5)	0 (0.0)	0 (0.0)	1 (0.5)	138 (69.0)	3 (1.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	Negative control (D.W.) 0	100	0	1	0	0	0	1	1	100	100	0	0	0
			100	4	1	0	0	0	5	0		100	3	0	3
			200	4 (2.0)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	6 (3.0)	1 (0.5)		200	3 (1.5)	0 (0.0)	3 (1.5)
6 - 18	+	868	100	0	0	0	0	0	0	0	72.1	100	0	0	0
			100	1	2	0	0	0	3	0	75.0	100	0	0	0
			200	1 (0.5)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.5)	0 (0.0)	(73.6)	200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	1040	100	1	7	0	0	0	7	0	62.8	100	0	0	0
			100	2	11	0	0	0	9	0	58.9	100	0	0	0
			200	3 (1.5)	18 (9.0)	0 (0.0)	0 (0.0)	0 (0.0)	16 (8.0)	0 (0.0)	(60.9)	200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	1250	100	6	27	1	0	0	19	0	48.7	100	0	0	0
			100	8	22	0	0	0	16	0	46.4	100	0	0	0
			200	14 (7.0)	49 (24.5)	1 (0.5)	0 (0.0)	0 (0.0)	35 (17.5)	0 (0.0)	(47.6)	200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	Positive control (CPA) 6	100	50	74	0	0	0	64	0		100	0	0	0
			100	44	56	0	0	0	51	2		100	0	0	0
			200	94 (47.0)	130 (65.0)	0 (0.0)	0 (0.0)	0 (0.0)	115 (57.5)	2 (1.0)		200	0 (0.0)	0 (0.0)	0 (0.0)

Treatment time comprised treatment-time and recovery-time.

The number of aberrant cells at each dish was shown at the first and the second lines. The total number of them was shown at the third line.

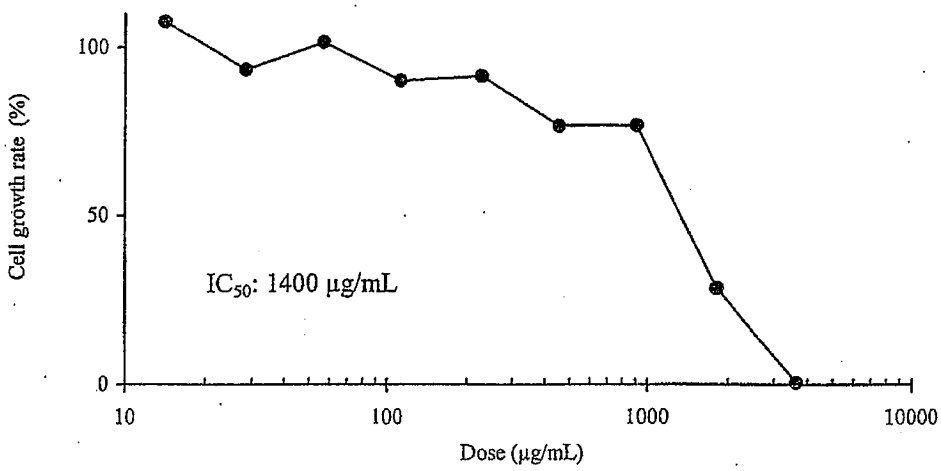
Cell growth rate at each dish was shown at the first and the second lines. The average of them was shown at the third line.

Multiple was shown at others in structural chromosomal aberrations.

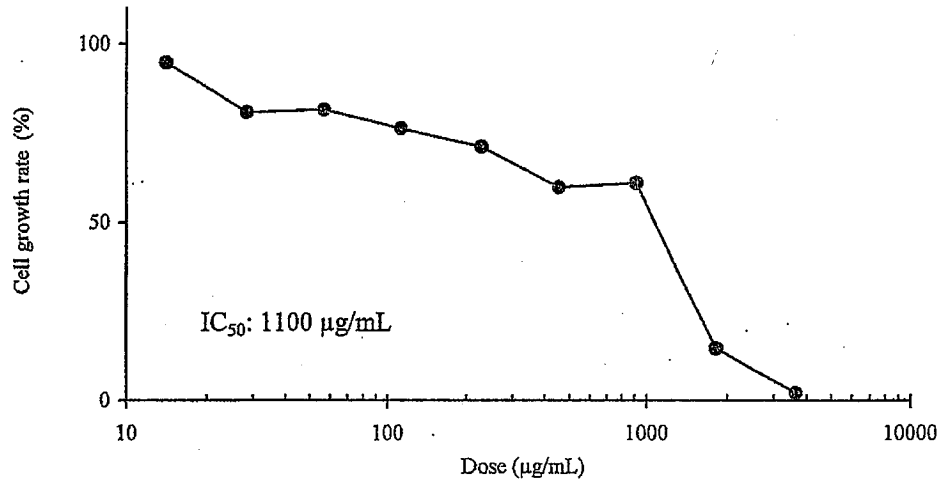
D.W.: Distilled water

MMC: Mitomycin C

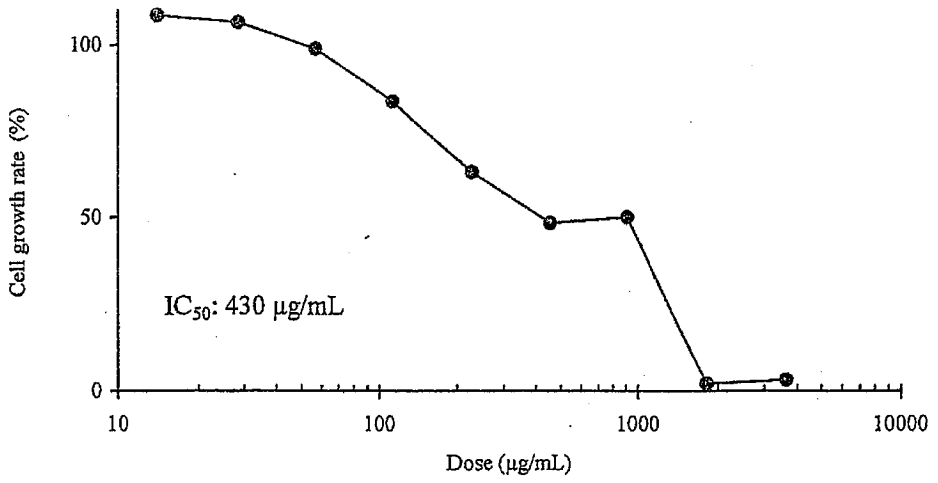
CPA: Cyclophosphamide monohydrate



Short-term treatment without S9 mix

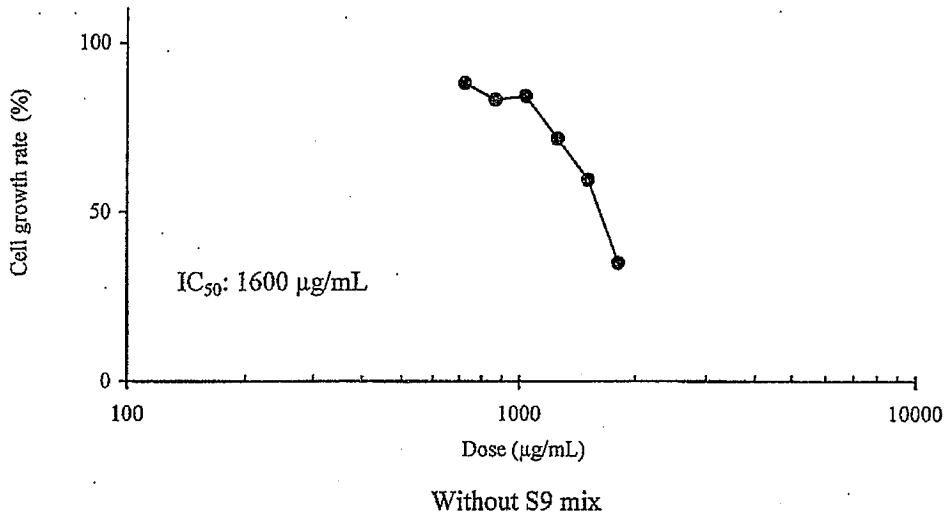


Short-term treatment with S9 mix

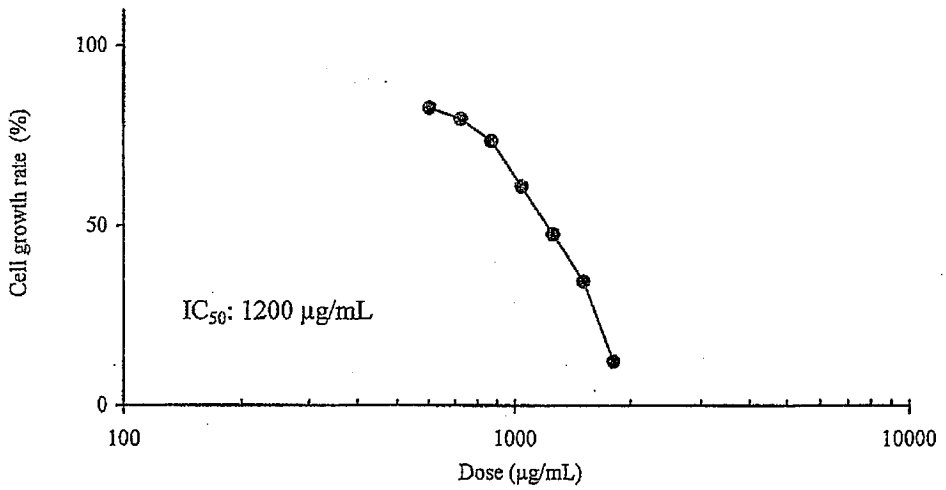


24 hours continuous treatment

Fig. 1 Results of cell growth inhibition test

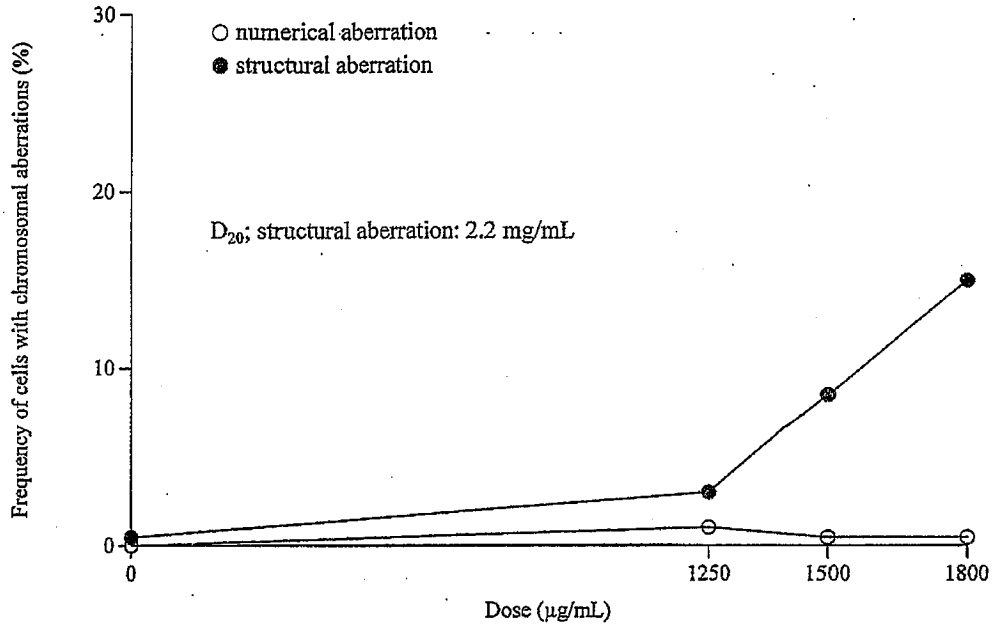


Without S9 mix

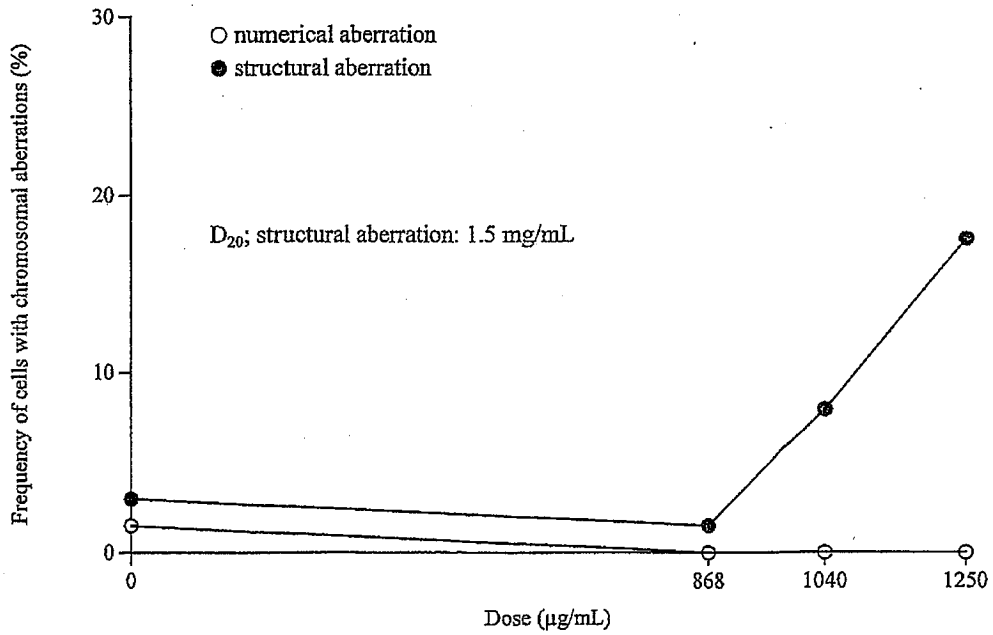


With S9 mix

Fig. 2 Cell growth rate for short-term treatments in chromosomal aberration test



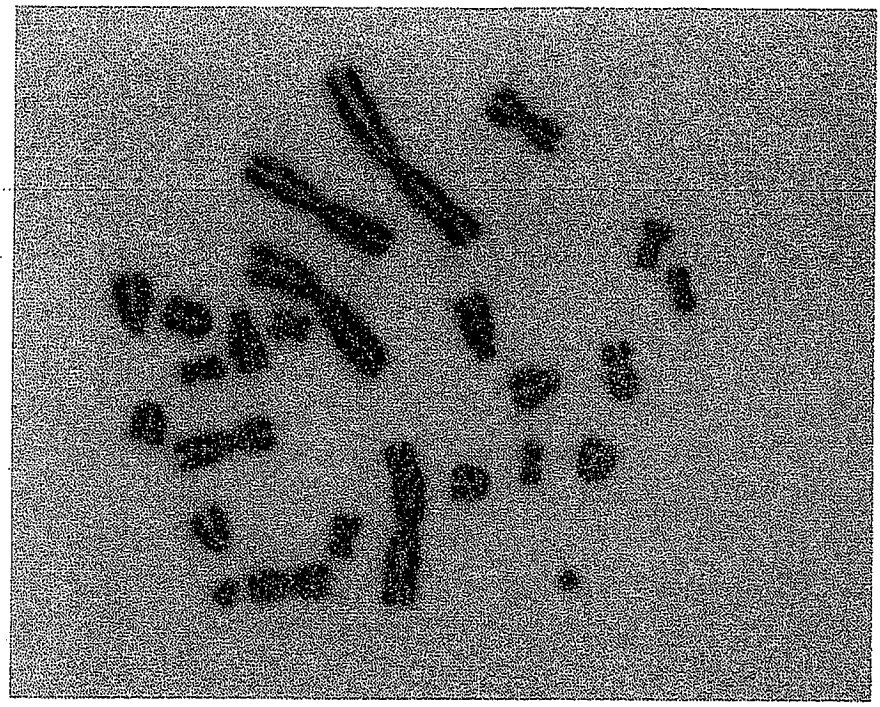
Without S9 mix



With S9 mix

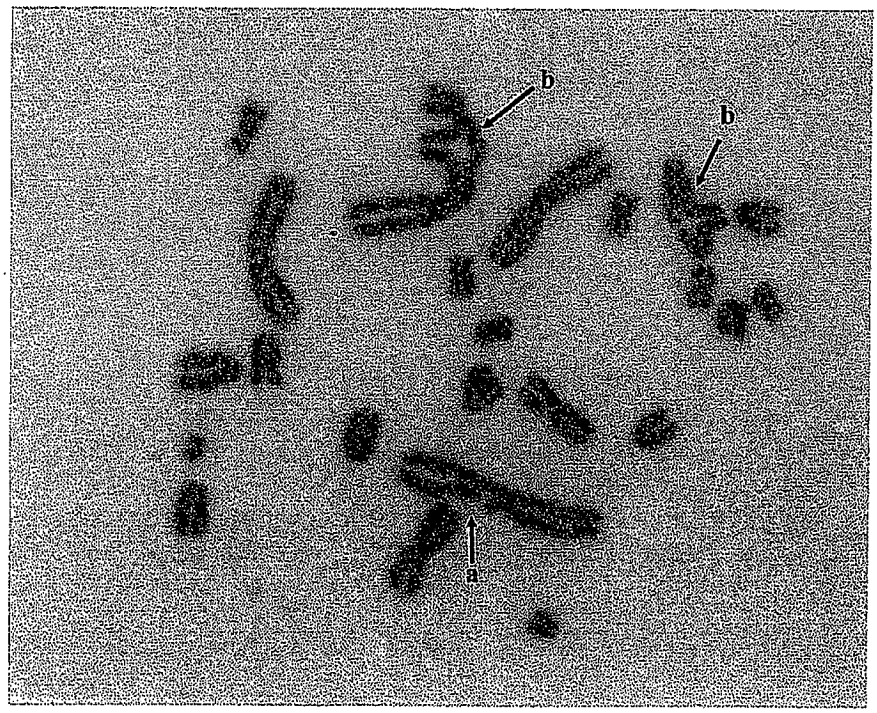
Fig. 3 Results of chromosomal aberration test in short-term treatments

Photo 1 Normal cell



Short-term treatment without S9 mix Negative control

Photo 2 Structural aberrations induced by



Short-term treatment without S9 mix 1800 μg/mL

a : Chromatid break b : Chromatid exchange

APPENDIX 1.

RESULTS OF OBSERVATION OF CHROMOSOMES IN ACCORDANCE WITH "CONCERNING TESTING METHODS RELATING TO THE NEW CHEMICAL SUBSTANCES"

Table 1 Results of chromosomal aberration test (short-term treatment)

Name of test substance :

K06-1120

Treatment time (h)	S9 mix	Dose (μg/mL)	Number of cells with structural chromosomal aberrations (frequency%)							Number of gaps (frequency%)	Cell growth rate (%)	Number of cells with numerical chromosomal aberrations (frequency%)			
			Number of cells observed	Chromatid break	Chromatid exchange	Chromosome break	Chromosome exchange	Others	Total number of cells with aberrations			Number of cells observed	Polyploids	Others	Total number of cells with aberrations
6 - 18	−	Negative control (D.W.) 0	100	0	0	0	0	0	0	2	100	100	0	0	0
			100	1	0	0	0	0	1	0		100	0	0	0
			200	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	2 (1.0)		200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	−	1250	100	0	1	0	0	0	1	0	72.1	100	1	0	1
			100	4	0	0	1	0	5	0	71.2	100	1	0	1
			200	4 (2.0)	1 (0.5)	0 (0.0)	1 (0.5)	0 (0.0)	6 (3.0)	0 (0.0)	(71.7)	200	2 (1.0)	0 (0.0)	2 (1.0)
6 - 18	−	1500	100	6	6	0	0	0	12	0	55.3	100	0	0	0
			100	3	3	0	0	0	5	0	63.9	100	1	0	1
			200	9 (4.5)	9 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)	17 (8.5)	0 (0.0)	(59.6)	200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	−	1800	100	5	8	0	0	0	11	0	31.2	100	0	0	0
			100	11	11	1	0	0	19	0	39.0	100	1	0	1
			200	16 (8.0)	19 (9.5)	1 (0.5)	0 (0.0)	0 (0.0)	30 (15.0)	0 (0.0)	(35.1)	200	1 (0.5)	0 (0.0)	1 (0.5)
6 - 18	−	Positive control (MMC) 0.1	100	35	55	0	0	1	71	1		100	0	0	0
			100	39	48	0	0	0	67	2		100	0	0	0
			200	74 (37.0)	103 (51.5)	0 (0.0)	0 (0.0)	1 (0.5)	138 (69.0)	3 (1.5)		200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	Negative control (D.W.) 0	100	0	1	0	0	0	1	1	100	100	0	0	0
			100	4	1	0	0	0	5	0		100	3	0	3
			200	4 (2.0)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	6 (3.0)	1 (0.5)		200	3 (1.5)	0 (0.0)	3 (1.5)
6 - 18	+	868	100	0	0	0	0	0	0	0	72.1	100	0	0	0
			100	1	2	0	0	0	3	0	75.0	100	0	0	0
			200	1 (0.5)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (1.5)	0 (0.0)	(73.6)	200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	1040	100	1	6	0	0	0	7	0	62.8	100	0	0	0
			100	2	7	0	0	0	9	0	58.9	100	0	0	0
			200	3 (1.5)	13 (6.5)	0 (0.0)	0 (0.0)	0 (0.0)	16 (8.0)	0 (0.0)	(60.9)	200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	1250	100	6	15	1	0	0	19	0	48.7	100	0	0	0
			100	7	13	0	0	0	16	0	46.4	100	0	0	0
			200	13 (6.5)	28 (14.0)	1 (0.5)	0 (0.0)	0 (0.0)	35 (17.5)	0 (0.0)	(47.6)	200	0 (0.0)	0 (0.0)	0 (0.0)
6 - 18	+	Positive control (CPA) 6	100	38	44	0	0	0	64	0		100	0	0	0
			100	32	37	0	0	0	51	2		100	0	0	0
			200	70 (35.0)	81 (40.5)	0 (0.0)	0 (0.0)	0 (0.0)	115 (57.5)	2 (1.0)		200	0 (0.0)	0 (0.0)	0 (0.0)

Treatment time comprised treatment-time and recovery-time.

The number of aberrant cells at each dish was shown at the first and the second lines. The total number of them was shown at the third line.

Cell growth rate at each dish was shown at the first and the second lines. The average of them was shown at the third line.

Multiple was shown at others in structural chromosomal aberrations.

D.W.: Distilled water

MMC: Mitomycin C

CPA: Cyclophosphamide monohydrate

APPENDIX 2

BACKGROUND DATA IN THE TESTING FACILITY

BACKGROUND DATA IN THE TESTING FACILITY

Negative control

Treatment method		Frequency of cells with chromosomal aberrations (%, mean±S.D.)	
		Structural aberration	Numerical aberration
Short-term treatment	Without S9 mix	1.0 ±0.78	0.7 ±0.59
	With S9 mix	1.1 ±0.67	0.7 ±0.64

Treatment method		Range of frequency of cells with chromosomal aberrations (%, mean±3S.D.)	
		Structural aberration	Numerical aberration
Short-term treatment	Without S9 mix	0.0 ~ 3.3	0.0 ~ 2.5
	With S9 mix	0.0 ~ 3.1	0.0 ~ 2.6

When the minimum range was below 0, it was shown "0.0".

Positive control

Treatment method		Substance	Dose (μg/mL)	Frequency of cells with chromosomal aberrations (%, mean±S.D.)	
				Structural aberration	Numerical aberration
Short-term treatment	Without S9 mix	MMC	0.1	53.4 ±7.49	0.6 ±0.36
	With S9 mix	CPA	6	44.2 ±18.49	0.4 ±0.37

Treatment method		Range of frequency of cells with chromosomal aberrations (%, mean±2S.D.)	
		Structural aberration	Numerical aberration
Short-term treatment	Without S9 mix	38.4 ~ 68.4	0.0 ~ 1.3
	With S9 mix	7.2 ~ 81.2	0.0 ~ 1.1

When the minimum range was below 0, it was shown "0.0".

Newest 20 test data were completed before November 4, 2005.